How to maintain active zone integrity during high-frequency transmission

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Abstract: In the central nervous system, the frequency at which reliable synaptic transmission can be maintained varies strongly between different types of synapses. Several pre- and postsynaptic processes must interact to enable high-frequency synaptic transmission. One of the mechanistically most challenging issues arises during repetitive neurotransmitter release, when synaptic vesicles fuse in rapid sequence with the presynaptic plasma membrane within the active zone (AZ), potentially interfering with the structural integrity of the AZ itself. Here we summarize potential mechanisms that help to maintain AZ integrity, including arrangement and mobility of release sites, calcium channel mobility, as well as release site clearance via lateral diffusion of vesicular proteins and via endocytotic membrane retrieval. We discuss how different types of synapses use these strategies to maintain high-frequency synaptic transmission.

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Review article

How to maintain active zone integrity during high-frequency transmission

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ABSTRACT

In the central nervous system, the frequency at which reliable synaptic transmission can be maintained varies strongly between different types of synapses. Several pre- and postsynaptic processes must interact to enable high-frequency synaptic transmission. One of the mechanistically most challenging issues arises during repetitive neurotransmitter release, when synaptic vesicles fuse in rapid sequence with the presynaptic plasma membrane within the active zone (AZ), potentially interfering with the structural integrity of the AZ itself. Here we summarize potential mechanisms that help to maintain AZ integrity, including arrangement and mobility of release sites, calcium channel mobility, as well as release site clearance via lateral diffusion of vesicular proteins and via endocytotic membrane retrieval. We discuss how different types of synapses use these strategies to maintain high-frequency synaptic transmission.

Contents

1. Introduction

Signal transmission via chemical synapses is integral to the function of the nervous system. During synaptic transmission, a presynaptic action potential (AP) causes brief opening of voltage-gated calcium (Ca^{2+}) channels. The resulting influx of Ca^{2+} ions then triggers the fusion of neurotransmitter-filled synaptic vesicles with the presynaptic membrane within the so-called active zone (AZ; Kaeser and Regehr, 2014; Schoch and Gundelfinger, 2006; Südhof, 2012). As a result, the membrane and proteins of synaptic vesicles are incorporated into the plasma membrane within the AZ. Assuming a diameter of 200 nm for the AZ (Holderith et al., 2012; Schikorski and Stevens, 1997) and a diameter of 40 nm for a synaptic vesicle (Hu et al., 2008), the fusion of only three vesicles would enlarge the AZ area by 50%. Based on larger estimates of vesicle size (80 aF/vesicle; Sakaba, 2006; and 1 $\mu F\,cm^{-2}$; Gentet et al., 2000), the fusion of only two vesicles would be sufficient to increase the AZ area by even 60%. Thus, vesicle fusion instantaneously alters AZ structure, thereby potentially impairing the integrity of AZ constitution and function. For example, the coupling between Ca^{2+} channels and Ca^{2+} sensor for vesicle fusion could be impaired or

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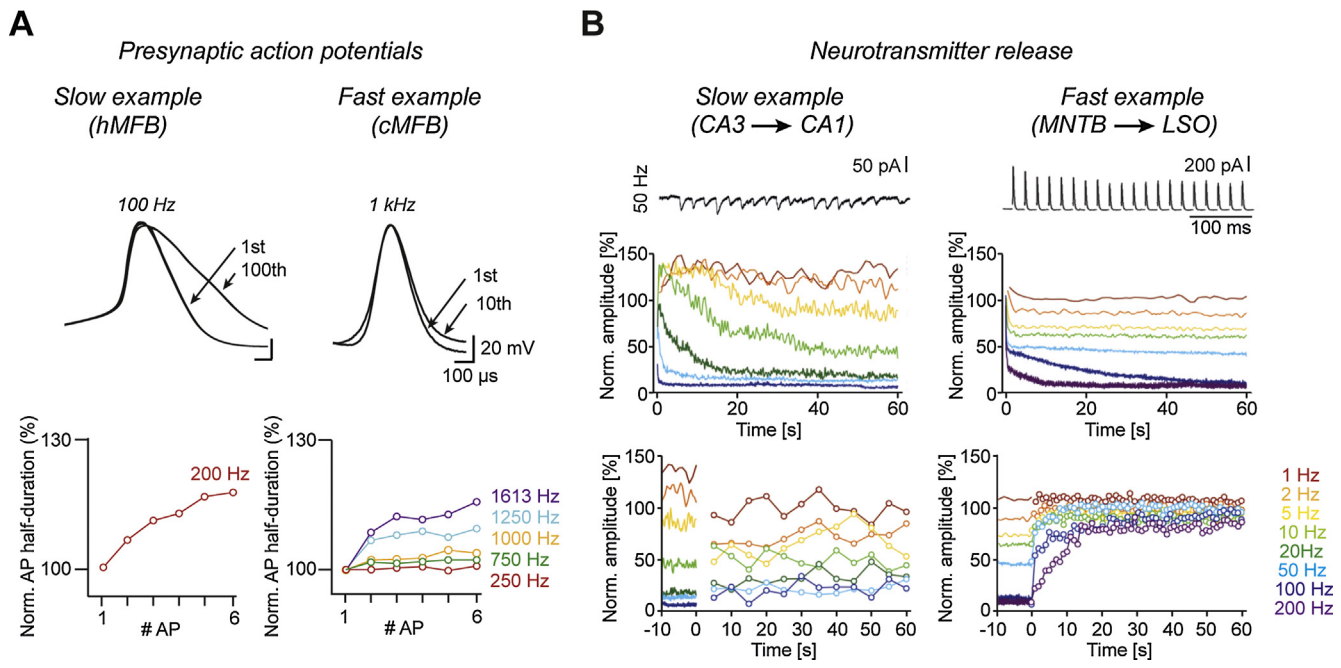

Fig. 1. Differential performance during high-frequency transmission.
(A) Comparison of AP waveforms during repetitive firing at two presynaptic boutons: The "slow-firing" hippocampal mossy fiber bouton (hMFB, left) and the "fast-firing" cerebellar mossy fiber bouton (cMFB, right). Top: Superposition of first and last presynaptic AP recorded in hMFBs and cMFBs during train-stimulation at 100 Hz (hMFB) and 1 kHz (cMFB). Bottom: Normalized AP half-durations during trains of six stimuli at indicated frequencies. (Left, from: Geiger and Jonas, 2000; right: unpublished data from I.D. (top) and from Ritzau-Jost et al., 2014 (bottom)).
(B) Differential synaptic performance during high-frequency transmission, exemplified at the "slow" glutamatergic CA3 to CA1 pyramidal cell synapse in the hippocampus (left) and at the "fast" glycinergic medial nucleus of the trapezoid body to lateral superior olive synapse (MNTB-LSO, right). Top: Postsynaptic inward (CA3-CA1) and outward (MNTB-LSO) currents evoked by 50 Hz stimulation indicate different levels of short-term synaptic plasticity. Middle: Normalized amplitudes of postsynaptic currents evoked at different frequencies (indicated by color code in lower panel). Bottom: Recovery time course of postsynaptic currents following train stimulation (see color code in right panel; from: Krächan et al., 2017).

the protein-protein interactions of the release machinery of the remaining vesicles could be disrupted. Furthermore, it has been suggested that the site where a vesicle fused is in a refractory state for many seconds, unable to offer "a competent fusion molecule" to the next approaching vesicle (Betz, 1970; Katz, 1996). Before a new vesicle can fuse at the site of a preceding fusion event, this site must consequently be cleared from, e.g., vesicular proteins – a process now referred to as release site clearance (Neher, 2010, 2017).

The frequency at which synapses operate varies strongly between different types of synaptic connections in the mammalian CNS (Delvendahl and Hallermann, 2016). For example, hippocampal mossy fiber boutons (hMFBs) usually operate at frequencies <100 Hz and show strong AP broadening, i.e. an activity-dependent prolongation of the AP duration due to, e.g., the inactivation of voltage-dependent potassium channels (Geiger and Jonas, 2000). On the other hand, cerebellar mossy fiber boutons (cMFBs) operate at frequencies up to 1 kHz and show only little AP broadening, most likely representing a special adaptation to the high frequencies that these presynaptic terminals operate at (Fig. 1A; Jörntell and Ekerot, 2006; Rancz et al., 2007; Ritzau-Jost et al., 2014).

The reliability of neurotransmitter release from synaptic vesicles, driven by the presynaptic release machinery, is even more variable across different types of synapses (Atwood and Karunanithi, 2002). For example, at synapses between hippocampal pyramidal cells, stimulation at frequencies exceeding 10 Hz leads to strong depression of synaptic transmission. In contrast, glycinergic synapses in the auditory brainstem maintain steady-state amplitudes during stimulation more effectively (Fig. 1B; Krächan et al., 2017). These synapses also recover faster from depression after cessation of the stimulus compared to the hippocampal ones (Fig. 1B, lower panels). Hence distinct types of synapses exhibit a strikingly different frequency dependence of synaptic transmission and consequently different bandwidths of synaptic transmission.

The structural and molecular differences underlying the functional diversity of synapses are currently poorly understood. Impairment of AZ integrity is likely a critical parameter determining the frequency-dependence of synaptic transmission. However, there are several other factors that can limit transmission frequency, such as onset kinetics of postsynaptic receptor saturation and receptor desensitization, the recovery from saturation/desensitization, and/or transmitter clearance from the synaptic cleft. Furthermore, a variety of presynaptic processes contribute to setting the maximum transmission frequency, such as intracellular Ca^{2+} accumulation (Delvendahl et al., 2015; Helmchen et al., 1997), vesicle mobility (Rothman et al., 2016), and vesicle recruitment (Hallermann and Silver, 2013). In this review, we focus on the integrity of the AZ during high-frequency synaptic transmission and identify five strategies that help to maintain AZ integrity. The discussion is based on full-collapse fusion of vesicles in order to limit the scope of this review. We note, however, that transient fusion-pore opening (kiss-and-run) of synaptic vesicles could represent a powerful strategy to maintain AZ integrity (Zhang et al., 2009).

2. Strategies to maintain AZ integrety

For the subsequent discussion, we would like to define the following four terms first (see Fig. 2).

1. We refer to a **synapse** as a chemical synapse, a single neuronal connection between a presynaptic en passant bouton or a ter-

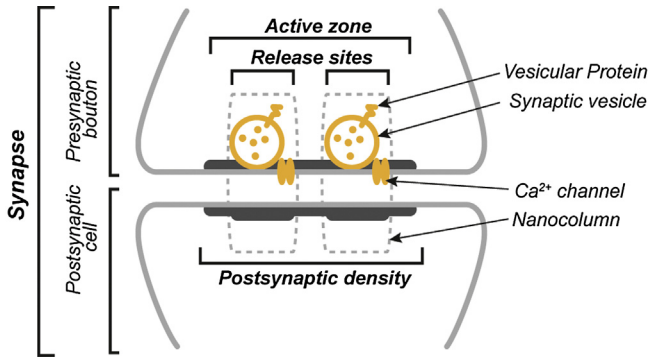

Fig. 2. Definition of the terms synapse, AZ, release site, and nanocolumn.
Schematic illustration of a synapse, consisting of a presynaptic bouton and a postsynaptic compartment (for a detailed elaboration of terms and concepts see main text): A presynaptic bouton contains one or several AZs, each opposing a postsynaptic density. Each AZ can harbor one or more release sites, defined as slots within AZs where vesicles can fuse. Recent findings suggest that pre- and postsynaptic structures are aligned in so-called nanocolumns.

minal and a postsynaptic compartment (e.g., a dendritic spine or postsynaptic soma).

2. The **AZ** is the anatomical structure appearing in electron microscopy as an electron dense structure opposing the postsynaptic density (Südhof, 2012). The number of AZs per synapse varies significantly between different types of synapses. For example, boutons of cortical pyramidal cells contain only a single AZ (Koester and Johnston, 2005; Silver et al., 2003), whereas the calyx of Held — a giant presynaptic terminal in the auditory brainstem (Forsythe, 1994; von Gersdorff and Borst, 2002) — features several hundred AZs (Sätzler et al., 2002).
3. A **release site** is the site where a vesicle can fuse. The structural constituents of release sites are currently not sufficiently understood, but recent studies indicate that within the AZ each release site contains a separate cluster of Ca^{2+} channels (Miki et al., 2017) and a separate Munc13-1 nano-assembly (Sakamoto et al., 2017). The number of release sites is usually determined functionally by the maximum number of vesicles that can be released. This value is identical to the pool of release-ready vesicles (RRP; Delvendahl and Hallermann, 2016; Kaeser and Regehr, 2017; Neher, 2015). However, if the occupancy of release sites is <1 (Malagon et al., 2016), the number of release sites is larger than the RRP (Pulido and Marty, 2017).
4. Finally, **'nanocolumns'** have been described as a precise alignment of presynaptic clusters of Rab3-interacting molecule (RIM) with clusters of postsynaptic receptors (Tang et al., 2016), raising the question if the number of nanocolumns corresponds to the number of release sites.

2.1. Strategy I: high number of release sites

Because multiple AZs and release sites can be present per synapse, the frequency of transmitter release at an entire given synapse is not necessarily identical to the frequency of release at individual release sites. Thus, depending on the vesicular release probability (p_r), the usage of individual release sites can be low, but parallel release at multiple release sites adds up to a high overall synaptic transmission frequency. Hence one of the most obvious strategies to maintain AZ integrity during sustained transmission is the parallel usage of many release sites (Fig. 3A), each exhibiting a low p_r.

A synapse that seems to adopt this strategy is the calyx of Held, which features a large RRP of ~1500 vesicles (Borst and Soria van Hoeve, 2012; Neher and Sakaba, 2008). Due to a low p_r, only ~50 of these vesicles are released per AP. High overall transmission

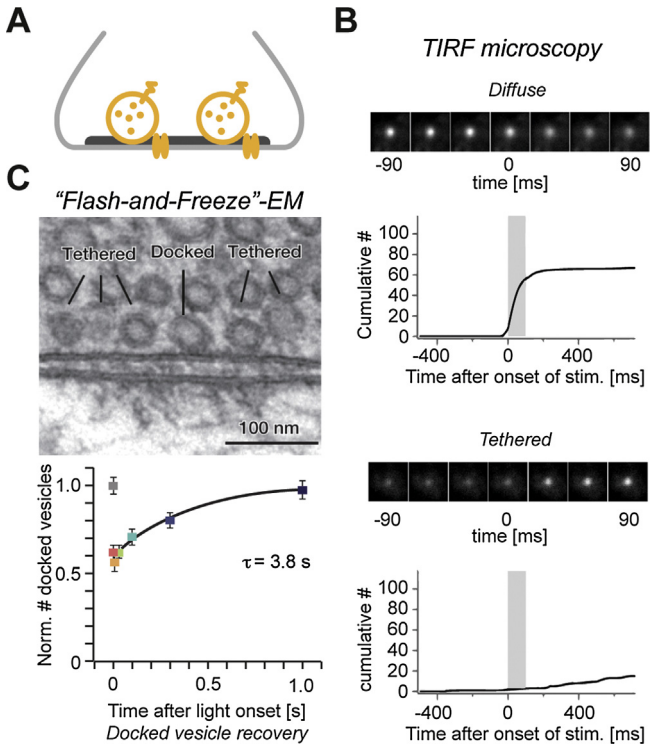

Fig. 3. Strategy I: Parallel use of multiple release sites.
(A) Illustration of an AZ with two release sites.
(B) Visualization of FM-labeled vesicles from dissociated presynaptic terminals by TIRF microscopy. Fluorescence changes were classified into two groups: 'Diffuse' events showed diminishing of fluorescence after application of a depolarizing stimulus by spreading of the fluorescence into the surrounding area (top). In contrast, 'tethered' events, once they emerged, were stationary until the end of a given recording period (bottom). Cumulative plots of peristimulus histograms for both types of events are shown: 'Diffuse' events predominantly occurred during the stimulation period and therefore likely represent stimulus triggered exocytosis of release-ready, FM-dye-labeled vesicles. 'Tethered' events were primarily detected after the onset of depolarization, indicating tethering of new vesicles following exocytosis (from: Midorikawa and Sakaba, 2015).
(C) Top: Electron micrograph of tethered and docked vesicles (tethered ones are closely associated, whereas docked ones are in direct proximity and visually bound to the plasma membrane). Bottom: Recovery of docked vesicles after optogenetically induced AP-driven exocytosis. Following stimulation, cells were rapidly frozen under high-pressure. The graph shows the number of docked vesicles at defined time points after light onset, normalized to the non-stimulated control (gray) (Watanabe et al., 2013b).

rates can thus be sustained with low-frequent release site activity. Indeed, a recent study using total internal reflection fluorescence (TIRF) microscopy at the calyx of Held showed that recruitment of new vesicles into the TIRF field requires several seconds (Fig. 3B; Midorikawa and Sakaba, 2015), indicating a very large RRP with a long average recruitment time for a new vesicle (~3 s). However, the resolution of TIRF microscopy (Brunstein et al., 2014) might not allow dissection of docking and tethering (Imig et al., 2014). Interestingly, a similar recruitment time constant (~4 s) has been observed at hippocampal synapses, where the recruitment of docked vesicles after optogenetically induced AP-driven exocytosis was determined by 'flash-and-freeze' electron microscopy (Fig. 3C; Watanabe et al., 2013b).

In contrast, cMFBs are capable of high-frequent synaptic transmission comparable to the calyx of Held (Delvendahl and Hallermann, 2016) but rely on a small pool of high p_r vesicles that can be recruited rapidly (Ritzau-Jost et al., 2014; Saviane and Silver, 2006). Furthermore, at the *Drosophila* neuromuscular junction, a subset of release sites is preferentially used during high-frequency transmission (Melom et al., 2013; Peled et al., 2014) and at the vertebrate neuromuscular junction, 'hot spots' for release have been

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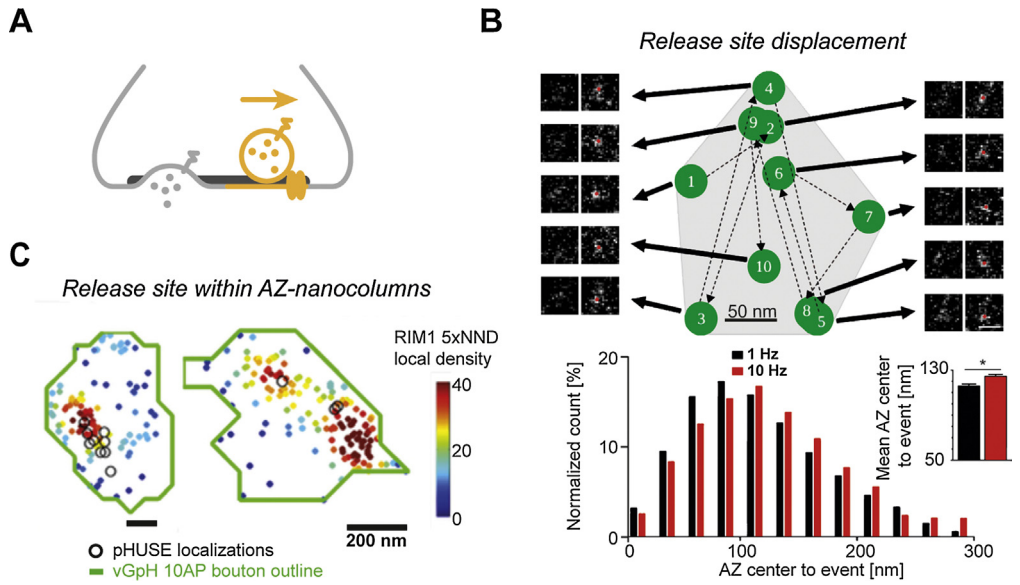

Fig. 4. Strategy II: Release site movement.
(A) Illustration of the lateral movement of an entire release site due to fusion of a neighboring vesicle.
(B) Top: Ten consecutive single vesicle fusion events (green circles, 1 Hz stimulation) within a single AZ (gray area) recorded by single-particle imaging of pHlourin-tagged vGlut1 in a hippocampal culture preparation. Images before (left) and after (right) fusion are shown for each single fusion event; red dots mark sub-pixel event localizations. Bottom: Distribution of distance of release event to AZ center for stimulation at 1 Hz and 10 Hz (in black and red, respectively). Inset: mean distance of release events to AZ center for 1 and 10 Hz stimulation (Maschi and Klyachko, 2017).
(C) Distribution of single fusion events of pHluorin-labeled vesicles (circles) and mEOS3-tagged RIM1 (colored dots) in AZs (green lines) of cultured hippocampal neurons. Color code indicates RIM1 density quantified as fluorescence within 5 x the standard deviation of nearest neighbor distances (NND) of RIM1 locations (Tang et al., 2016).

demonstrated (Gaffield et al., 2009), which seems to contradict the idea of parallel usage of many release sites. Thus, the strategy of using many release sites per synapse in parallel seems to be employed by only some types of synapses.

2.2. Strategy II: release site movements

Vesicle fusion and incorporation into the presynaptic membrane necessarily extend the plasma membrane surface, potentially threatening release site integrity or displacing neighboring release sites laterally away from the site of the release event (Fig. 4A). In theory, displacement could provide a strategy to maintain release site integrity, because entire release sites might only be shifted but otherwise not impaired.

Although this scenario remains hypothetical to date, recent technical developments might offer means to address this question. In particular, super-resolution imaging techniques (reviewed in: Dani and Huang, 2010; Ehmann et al., 2015; Maglione and Sigrist, 2013; Maidorn et al., 2016; Sigrist and Sabatini, 2012) enable detection of the position of vesicle fusion events within individual AZs (Maschi and Klyachko, 2017; Tang et al., 2016). For example, Maschi and Klyachko (2017) revealed the presence of multiple distinct release sites within individual hippocampal synapses that usually contain only a single AZ (Fig. 4B, upper panel). They showed re-use of release sites during 1 Hz activity and determined a lower-bound release site recovery time of at least 100 ms, which is in good agreement with estimates based on TIRF microscopy (Midorikawa and Sakaba, 2015). Furthermore, the mean distance of a fusion site to the center of the AZ increased during stimulation at 10 Hz compared to 1 Hz (Fig. 4B, lower panel). A preferential engagement of more peripherally located release sites could be due to low-p_r release sites at the edge of the AZ. Indeed, recent studies at the *Drosophila* neuromuscular junction revealed remote low-p_r release sites containing the AZ protein Unc13B (Böhme et al., 2016; Reddy-Alla et al., 2017). Alternatively, the entire protein cluster comprising release machinery and Ca^{2+} channels may "drift" in the AZ plane due to fusion of neighboring vesicles. Such release site movement in the nm and ms spatio-temporal domain seems consistent with the high plasticity (Glebov et al., 2017) and mobility of AZ proteins (see next paragraph; Heine et al., 2008), but will be difficult to resolve and thus remains speculative. One complication of the release-site-displacement strategy would be a resulting misalignment of trans-synaptic nanocolumns (Figs. 2 and 4C; Tang et al., 2016). Dislocation of presynaptic release sites could therefore contribute to short-term depression of synaptic transmission.

2.3. Strategy III: Ca^{2+} channel mobility

One of the most critical parameters determining the reliability of neurotransmitter release is the distance between Ca^{2+} channels and the Ca^{2+} sensor governing vesicle fusion (reviewed in e.g. Eggermann et al., 2011; Neher, 1998; Stanley, 2016; Wang and Augustine, 2014). High mobility of Ca^{2+} channels within the AZ could allow them to diffuse towards vesicles that lost "their" Ca^{2+} channel due a recent perturbing fusion event (Fig. 5A). In other words, one could speculate that a more fluid and dynamic AZ structure is less "vulnerable" to a fusion event, because a fusion would have little impact on the subsequent p_r.

There is good evidence that the location of Ca^{2+} channels within AZs is not fixed. For example, N-terminal tagging of the α1 subunit of Ca^{2+} channels enabled direct measurements of Ca^{2+} channel mobility (Schneider et al., 2015; Fig. 5B,C). Furthermore, Ca^{2+} channel mobility seems to be activity-dependent (Mercer et al., 2011; Schneider et al., 2015; Stanley et al., 2003). For example, tracking the α2δ4 subunit with quantum dots indicated that depolarization-induced vesicle fusion causes presynaptic Ca^{2+} channels to be displaced laterally before rapidly moving back towards their initial position (Mercer et al., 2011). This process is governed by cholesterol and the actin cytoskeleton (Fig. 5D; Mercer et al., 2011; Saka et al., 2014; Taverna et al., 2004) and indicates that mobile Ca^{2+} channels may be able to leave currently refractory release sites. The dynamic arrangement of Ca^{2+} channels may optimize the density

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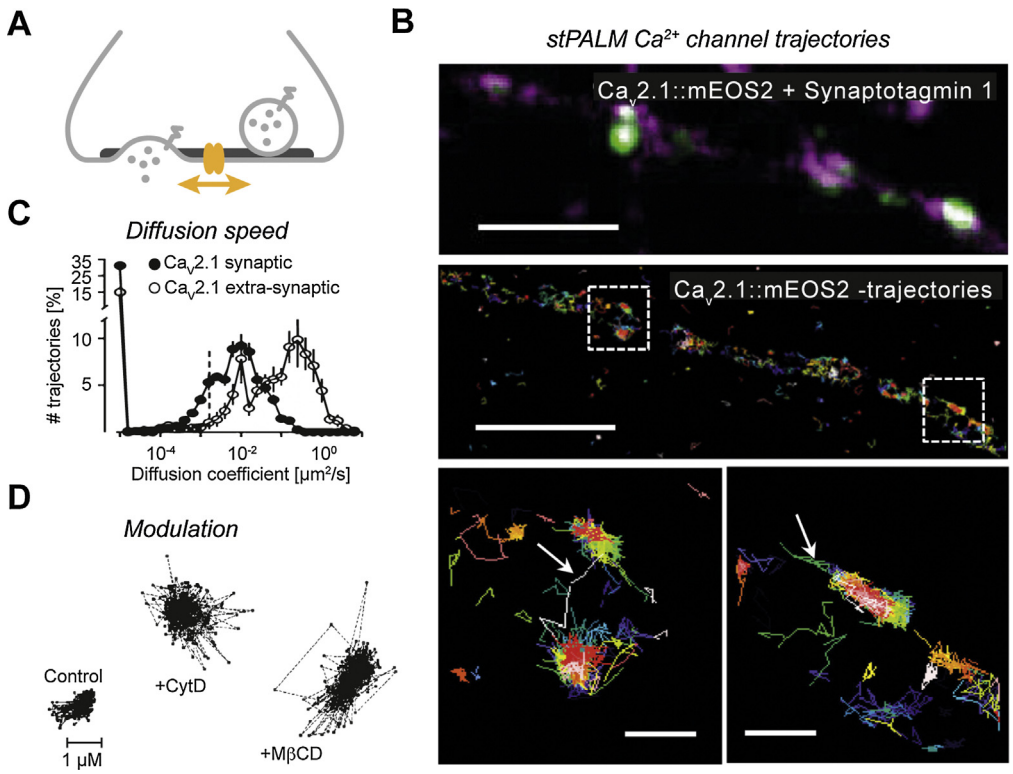

Fig. 5. Strategy III: Ca^{2+} channel mobility.
(A) Illustration of Ca^{2+} channel mobility.
(B) Top: Co-localization of mEOS2-tagged α1 Ca^{2+} channel subunits (green) and Synaptotagmin-1 (magenta) show that $Ca_V2.1$ Ca^{2+} channels are confined to presynaptic terminals. Middle: Mobility of $Ca_V2.1$ channel subunits assessed by single-particle tracking photoactivated localization microscopy (sptPALM). Trajectories of individual channel molecules along axonal segments are shown in different colors; selected regions are enlarged below. Channels are spatially confined, with only few channels escaping the synaptic compartment (arrows). Scale bars: 3 µm, enlarged axonal segments 0.5 µm.
(C) Normalized distribution of diffusion coefficients for mEOS2-tagged $Ca_V2.1$ channels. Dotted line represents threshold defined for immobile fractions. At synapses, 40% of the channels are immobile, whereas along axons only 20% of channels are classified as immobile. Within the mobile fraction, channels outside of synapses move faster than those confined to synapses (Data in (B) and (C) from: Schneider et al., 2015).
(D) Trajectories derived by tracking quantum dot-labeled α2δ-subunits of $Ca_V1.4$-type Ca^{2+} channels at dissociated photoreceptor synapses. Disrupting actin filaments or depleting cholesterol by application of cytochalasin-D (cytD) or methyl – β – cyclodextrin (MβCD), respectively, increased channel mobility as indicated by the enlarged displacement fields (from: Mercer et al., 2011).

of channels required for a given p_r. This way, Ca^{2+} channel mobility could act as an equalizer of p_r, thereby also affecting short-term synaptic plasticity (Heck et al., 2017; Schneider et al., 2015). However, nearly all data so far are derived from cultured neurons and future studies are required to analyze Ca^{2+} channel mobility *in vivo*.

2.4. Strategy IV: lateral movement of vesicular proteins

Vesicle fusion not only enlarges the presynaptic surface, but also incorporates vesicular proteins into the presynaptic plasma membrane. Subsequent removal of vesicular proteins from release sites is essential for maintaining release site function, because the accumulation of vesicular debris might jam a release site after use. Indeed, vesicular proteins, e.g., 'dead' cis-SNARE complexes, can interfere with the docking of new vesicles (Hosoi et al., 2009; Kim and von Gersdorff, 2009). Hence high mobility and rapid diffusion of vesicular proteins can be advantageous during sustained synaptic activity, clearing the way for subsequent fusion events (Fig. 6A).

The lateral movement or spread of vesicular proteins has, for example, been resolved in neuroendocrine cells by labeling the vesicular acetylcholine transporter (vAChT) with a pH-sensitive probe (pHluorin; Sochacki et al., 2012). At cultured hippocampal synapses, several proteins involved in vesicle release were shown to be highly mobile, such as the SNARE-protein syntaxin-1A (Schneider et al., 2015). Additionally, comparable lateral movement of pHluorin-tagged synaptobrevin-2 (Syb2), synaptotagmin-1 (Syt1) and synaptophysin (Syp) after AP-triggered exocytosis has been observed in these synapses (Fig. 6; Gimber et al., 2015).

Assuming diffusion coefficients between 0.20 and 0.35 $\mu m^2/s$ for Syb2, Syt1 and Syp, these vesicular proteins move ~1 µm away from the AZ within 2 s (Gimber et al., 2015). But unlimited diffusion out of the bouton and into the adjacent axon could cause difficulties in maintaining presynaptic (vesicle) protein levels. Consistently, after an initial phase of non-confined diffusion, Gimber et al. (2015) observed a restriction of free diffusion leading to a confinement of vesicular proteins within a 2–2.5 μm^2 sized area (Fig. 6). To date, the molecular identity of the structures surrounding the AZ and acting as short-range diffusion traps remains unknown. However, experiments performed on neuroendocrine cells and neuronal synapses indicate that endocytic assemblies, composed of a dense network of membrane-resident clathrin-coated structures, restrict the free diffusion of membrane proteins (Gimber et al., 2015; Sochacki et al., 2012).

In addition, the network of endocytic proteins also seems to play a role in re-clustering the previously dispersed proteins (Fig. 6). Clustering was observed, e.g., for fluorescently labeled synaptotagmin-1 that aggregates on the synaptic surface of cultured hippocampal neurons after stimulation (Willig et al., 2006). This clustering was later interpreted as formation of a "readily retrievable pool" (RRetP) of vesicles for compensatory endocytosis outside

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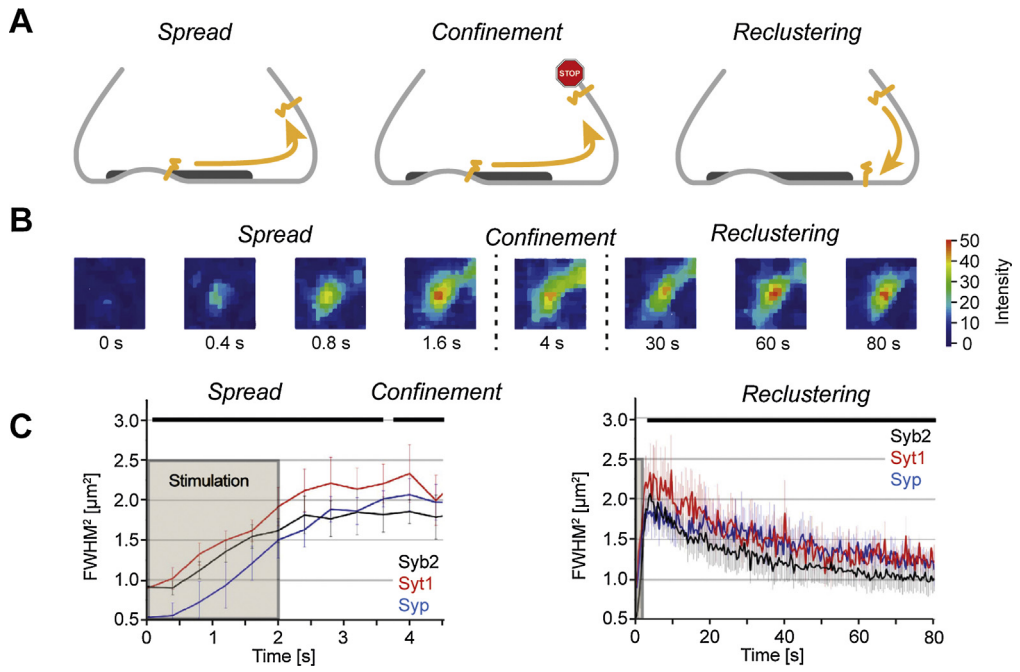

Fig. 6. Strategy IV: Lateral movement of vesicular proteins.
(A) Illustration of the initial spread, confinement, and reclustering of the vesicular proteins following exocytosis.
(B) Fluorescence dynamics of exocytosed pHluorin-tagged Syb2 following vesicle fusion evoked by 40 APs at 20 Hz. Syb2 initially diffuses rapidly away from fusion sites ('spread') indicating free diffusion, followed by a 'confinement' limiting diffusion to the bouton surface. Finally, a slow 'reclustering' is observed. Color code indicates fluorescence intensity.
(C) Full-width at half-maximum (FWHM) of Gaussian fits to fluorescence profiles observed for Syb2 (black), Syt1 (red) and Syp (blue) over time. Stimulation (as in B) leads to spreading and confinement (left) and is followed by reclustering (right) with comparable dynamics for all three proteins (Data in (B) and (C) from: Gimber et al., 2015).

the AZ, in an area termed periactive zone (Hua et al., 2011). Even though the exact mechanism governing translocation of proteins from release sites towards the RRetP remains elusive to date, a recent study identified the self-assembly of exocytosed Syb2 and Syp1 into oligomers as a key mechanism mediating release site clearance and clustering of proteins (Rajappa et al., 2016). Both vesicular proteins prevent cis-SNARE complex formation at the AZ and thereby short-term synaptic depression.

2.5. Strategy V: fast membrane and protein retrieval

Endocytosis is a central mechanism required to counterbalance exocytosis and to maintain release site functionality (Fig. 7A). Endocytosis is one of the latest steps in AZ clearance, but during sustained high-frequency transmission it might become rate-limiting (Kawasaki et al., 2000). Therefore, fast endocytosis seems to be a straightforward strategy for maintaining AZ integrity.

There is intense effort to determine the time course of endocytic membrane retrieval (Wu et al., 2014). Recently, optogenetic and electron-microscopic techniques revealed an ultrafast, clathrin-independent mode of membrane retrieval occurring at physiological temperatures (Fig. 7C; Watanabe et al., 2013a, 2013b). In addition, capacitance recordings in cerebellar and hippocampal MFBs resolved rapid membrane retrieval (Fig. 7B; Delvendahl et al., 2016). Furthermore, using quenching protocols and pHluorin–labeled Syp and Syt-1 (Fig. 7D), Soykan et al. (2017) demonstrated rapid protein uptake. The time course of this process was highly dependent on the stimulation protocol: Upon two APs, more than 50% of the exocytosed proteins were re-internalized rapidly within one second after stimulation, whereas stimulation with 10 APs reduced the share of ultrafast endocytosed proteins to 20-30% (Soykan et al., 2017). This resembles the dynamics of membrane retrieval in cerebellar and hippocampal MFBs, where stronger stimulation significantly decelerates endocytosis (Delvendahl et al., 2016). These observations suggest that the membrane area that can be retrieved in an ultrafast manner is limited (Thomas et al., 1994).

Surprisingly, endocytosis of presynaptic membrane and proteins does not necessarily share the same mechanisms (Fig. 7E). A recent study showed that, after inhibiting the Ca^{2+}-calmodulin-Munc13-1 pathway, uptake of fluorescently labeled synaptotagmin-2 was significantly impaired, whereas the uptake of presynaptic membrane, determined by simultaneous measurements of presynaptic membrane capacitance, was not affected (Okamoto et al., 2016). Although it is unclear if decoupling of membrane and protein retrieval plays a role under physiological conditions, these findings corroborate the idea that several distinct mechanisms of endocytosis act synergistically to mediate presynaptic membrane and protein retrieval. Several studies also indicate a direct link between endocytosis and synaptic performance. For example, quantification of RRetP size closely mirrored the size of the RRP at cultured hippocampal synapses (Wienisch and Klingauf, 2006), providing a potentially powerful mechanism preventing accumulation of vesicular proteins within the synaptic vesicle cycle (Rizzoli, 2014). In addition, interference with clathrin-mediated endocytosis, such as knockout of the GTPase Dynamin-1, can cause a decrease of synaptic depression, possibly due to an increased activity of compensatory rapid bulk endocytosis (Mahapatra et al., 2016).

3. Concluding remarks

Within the last two decades, several studies demonstrated that in order to sustain rapid vesicular release, synapses not only need to manage supply of release-competent vesicles, but also have to ensure release site availability by maintaining the integrity of the

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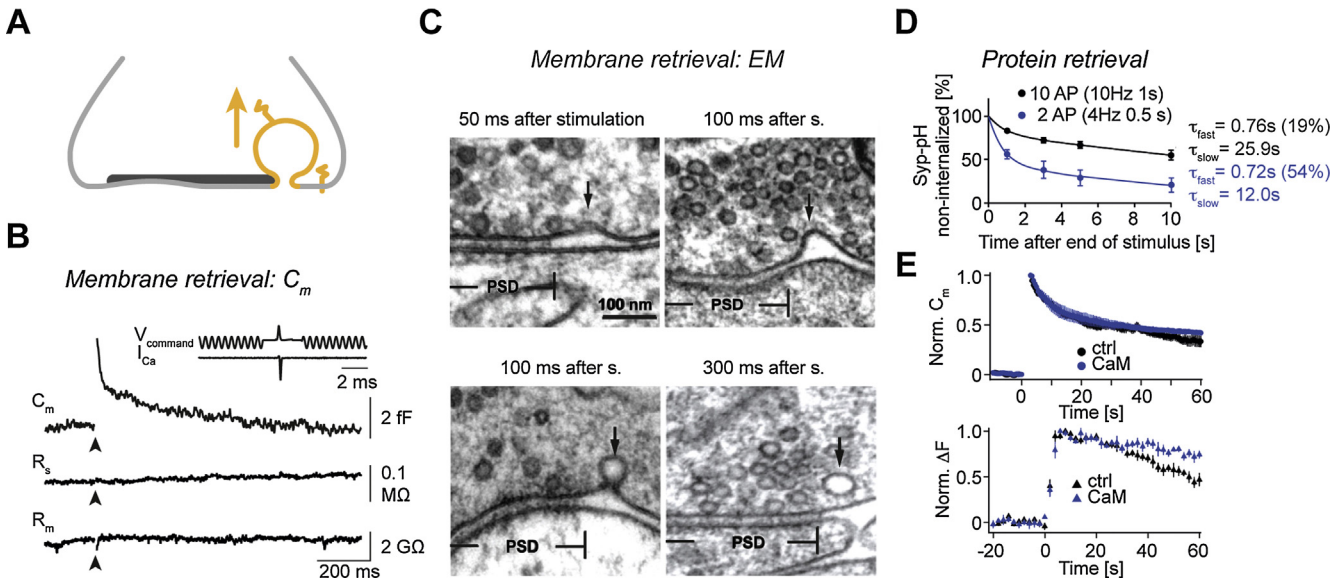

Fig. 7. Strategy V: Membrane and protein retrieval.
(A) Illustration of membrane and protein retrieval by endocytosis.
(B) Capacitance (C_m), series (R_s) and membrane resistance (R_m) of the cerebellar mossy fiber bouton in response to a single AP (arrow) measured at 36 °C. Inset: Voltage command ($V_{command}$) used for AP-evoked capacitance recordings and resulting Ca^{2+} current (I_{Ca}). The subsequent decay of C_m reflects ultrafast endocytosis with a time constant of ~500 ms (from: Delvendahl et al., 2016).
(C) Ultrafast endocytosis at physiological temperature using flash-and-freeze electron micrographs from presynaptic terminals of hippocampal neurons (cf. Fig. 3). Within several 100 ms after optogenetic stimulation, invaginations and large intraterminal vesicles appear (arrows) that are most prominent in the periactive zone (from: Watanabe et al., 2013b).
(D) Acid-induced quenching of pHluorin-labeled synaptophysin at different time points after stimulation reveals the time course of protein retrieval. In response to different stimulation protocols (10 Hz for 1 s vs. 4 Hz for 0.5 s), vesicle proteins are retrieved on multiple timescales at physiological temperature (from: Soykan et al., 2017).
(E) Comparison of slow membrane and protein retrieval modes and kinetics at the calyx of Held synapse using simultaneous capacitance recordings (C_m, left) and recordings of a pH-sensitive protein marker-tagged synaptotagmin-2 (CypHer, right). Intracellular application of a calmodulin inhibitory protein (CaM) does not disturb recovery of membrane capacitance but affects the recovery of the CypHer-signal, raising the possibility of two distinct endocytic pathways (from: Okamoto et al., 2016).

presynaptic AZ (Neher, 2010). In this review, we described five strategies (Fig. 3–7) adopted by synapses to preserve structure and functionality of the AZ. First, in order to reduce the average number of release events per site, the overall synaptic transmission rate can be maintained by parallel usage of several independent release sites (Fig. 3). Second, the drift of entire release sites away from sites of prior fusion events could be a mechanism to protect the integrity of a release site from disarrangement by the prior fusion events (Fig. 4). Third, high mobility of presynaptic Ca^{2+} channels could exert a stabilizing effect on presynaptic p_r, because fusion within a "fluid" AZ will have little effect on the average coupling distance between Ca^{2+} channels and Ca^{2+} sensor (Fig. 5). Fourth, diffusional spread and subsequent clustering of membrane-bound proteins has the potential to rapidly remove vesicular proteins from the center of the AZ (Fig. 6). Finally, fast modes of protein and membrane retrieval could help to accelerate the rearrangement of AZ structure (Fig. 7). The strategies IV and V represent release-site clearance and seem to be essential in order to avoid "traffic jams" (Kim and von Gersdorff, 2009), which could lead to synaptic depression during sustained activity (Hosoi et al., 2009).

Within the central nervous system, different types of chemical synapses coexist, each having evolutionarily adapted to perform distinctive tasks. Hence synapses display a huge diversity in composition, quantity, and location of the protein machinery responsible for neurotransmitter release. As outlined above, increasing evidence suggests that the differential performance of synapses during high-frequent activity arises at least in part from various degrees of AZ clearance and maintenance. Synapses probably implement each of the five strategies differently or to a varying degree, which may contribute to the diverse frequency bandwidths of synaptic transmission. Future studies are required to elucidate how mechanisms securing AZ integrity and processes governing vesicle supply (e.g., tethering, docking, and priming) cooperate to shape synaptic performance.

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